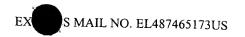# COMPOSITIONS AND METHODS FOR THE TREATMENT
AND DIAGNOSIS OF BREAST CANCER

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Patent Application No. 09/429,755, filed October 28, 1999, which is a continuation-in-part of U.S. Patent Application No. 09/289,198, filed April 9, 1999, which is a continuation-in-part of U.S. Patent Application No. 09/062,451, filed April 17, 1998, which is a continuation in part of U.S. Patent Application No. 08/991,789, filed December 11, 1997, which is a continuation-in-part of U.S. Patent Application No. 08/838,762, filed April 9, 1997, which claims priority from International Patent Application No. PCT/US97/00485, filed January 10, 1997, and is a continuation-in-part of U.S. Patent Application No. 08/700,014, filed August 20, 1996, which is a continuation-in-part of U.S. Patent Application No. 08/585,392, filed January 1, 1996.

TECHNICAL FIELD

The present invention relates generally to the detection and therapy of breast cancer. The invention is more specifically related to nucleotide sequences that are preferentially expressed in breast tumor tissue and to polypeptides encoded by such nucleotide sequences. The nucleotide sequences and polypeptides may be used in vaccines and pharmaceutical compositions for the prevention and treatment of breast cancer. The polypeptides may also be used for the production of compounds, such as antibodies, useful for diagnosing and monitoring the progression of breast cancer in a patient.

BACKGROUND OF THE INVENTION

Breast cancer is a significant health problem for women in the United States and throughout the world. Although advances have been made in detection and treatment of the disease, breast cancer remains the second leading cause of cancer-related deaths in women, affecting more than 180,000 women in the United States each year.

For women in North America, the life-time odds of getting breast cancer are now one in eight.

No vaccine or other universally successful method for the prevention or treatment of breast cancer is currently available. Management of the disease currently relies on a combination of early diagnosis (through routine breast screening procedures) and aggressive treatment, which may include one or more of a variety of treatments such as surgery, radiotherapy, chemotherapy and hormone therapy. The course of treatment for a particular breast cancer is often selected based on a variety of prognostic parameters, including an analysis of specific tumor markers. *See, e.g.*, Porter-Jordan and Lippman, *Breast Cancer 8*:73-100 (1994). However, the use of established markers often leads to a result that is difficult to interpret, and the high mortality observed in breast cancer patients indicates that improvements are needed in the treatment, diagnosis and prevention of the disease.

Accordingly, there is a need in the art for improved methods for therapy and diagnosis of breast cancer. The present invention fulfills these needs and further provides other related advantages.

SUMMARY OF THE INVENTION

Briefly stated, the subject invention provides compositions and methods for the diagnosis and therapy of breast cancer. In one aspect, isolated polynucleotides are provided, comprising (a) a nucleotide sequence preferentially expressed in breast cancer tissue, relative to normal tissue; (b) a variant of such a sequence, as defined below; or (c) a nucleotide sequence encoding an epitope of a polypeptide encoded by at least one of the above sequences. In one embodiment, the isolated polynucleotide comprises a human endogenous retroviral sequence recited in SEQ ID NO:1. In other embodiments, the isolated polynucleotide comprises a sequence recited in any one of SEQ ID NO: 3-26, 28-77, 142, 143, 146-152, 154-166, 168-176, 178-192, 194-198, 200-204, 206, 207, 209-214, 216, 218, 219, 221-240, 243-245, 247, 250, 251, 253, 255, 257-266, 268, 269, 271-273, 275, 276, 278, 280, 281, 284, 288, 291-298, 301-303, 307, 313, 314, 316 and 317.

In related embodiments, the isolated polynucleotide encodes an epitope of a polypeptide, wherein the polypeptide is encoded by a nucleotide sequence that: (a) hybridizes to a sequence recited in any one of SEQ ID NO: 1, 3-26, 28-77, 142, 143, 146-152, 154-166, 168-176, 178-192, 194-198, 200-204, 206, 207, 209-214, 216, 218, 219, 221-240, 243-245, 247, 250, 251, 253, 255, 257-266, 268, 269, 271-273, 275, 276, 278, 280, 281, 284, 288, 291-298, 301-303, 307, 313, 314, 316 and 317 under stringent conditions; and (b) is at least 80% identical to a sequence recited in any one of SEQ ID NO: 1, 3-26, 28-77, 142, 143, 146-152, 154-166, 168-176, 178-192, 194-198, 200-204, 206, 207, 209-214, 216, 218, 219, 221-240, 243-245, 247, 250, 251, 253, 255, 257-266, 268, 269, 271-273, 275, 276, 278, 280, 281, 284, 288, 291-298, 301-303, 307, 313, 314, 316 and 317.

In another embodiment, the present invention provides an isolated polynucleotide encoding an epitope of a polypeptide, the polypeptide being encoded by: (a) a nucleotide sequence transcribed from the sequence of SEQ ID NO: 141; or (b) a variant of said nucleotide sequence that contains one or more nucleotide substitutions, deletions, insertions and/or modifications at no more than 20% of the nucleotide positions, such that the antigenic and/or immunogenic properties of the polypeptide encoded by the nucleotide sequence are retained. Isolated DNA and RNA molecules comprising a nucleotide sequence complementary to a polynucleotide as described above are also provided.

In related aspects, the present invention provides recombinant expression vectors comprising a polynucleotide as described above and host cells transformed or transfected with such expression vectors.

In further aspects, polypeptides comprising an amino acid sequence encoded by a polynucleotide as described above, and monoclonal antibodies that bind to such polypeptides are provided. In certain embodiments, the inventive polypeptides comprise an amino acid sequence selected from the group consisting of SEQ ID NO: 299, 300, 304-306, 308 and 315, and variants thereof as defined below.

In yet another aspect, methods are provided for determining the presence of breast cancer in a patient. In one embodiment, the method comprises detecting, within a biological sample, a polypeptide as described above. In another embodiment, the

method comprises detecting, within a biological sample, an RNA molecule encoding a polypeptide as described above. In yet another embodiment, the method comprises (a) intradermally injecting a patient with a polypeptide as described above; and (b) detecting an immune response on the patient's skin and therefrom detecting the presence of breast

5 cancer in the patient. In further embodiments, the present invention provides methods for determining the presence of breast cancer in a patient as described above wherein the polypeptide is encoded by a nucleotide sequence selected from the group consisting of SEQ ID NO: 78-86, 144, 145, 153, 167, 177, 193, 199, 205, 208, 215, 217, 220, 241, 242, 246, 248, 249, 252, 256, 267, 270, 274, 277, 279, 282, 283, 285-287, 289, 290 and

10 sequences that hybridize thereto under stringent conditions.

In a related aspect, diagnostic kits useful in the determination of breast cancer are provided. The diagnostic kits generally comprise either one or more monoclonal antibodies as described above, or one or more monoclonal antibodies that bind to a polypeptide encoded by a nucleotide sequence selected from the group

15 consisting of sequences provided in SEQ ID NO: 78-86, 144, 145, 153, 167, 177, 193, 199, 205, 208, 215, 217, 220, 241, 242 and 246, 248, 249, 252, 256, 267, 270, 274, 277, 279, 282, 283, 285-287, 289, 290 and a detection reagent.

Diagnostic kits are also provided that comprise a first polymerase chain reaction primer and a second polymerase chain reaction primer, at least one of the

20 primers being specific for a polynucleotide described herein. In one embodiment, at least one of the primers comprises at least about 10 contiguous nucleotides of a polynucleotide as described above, or a polynucleotide encoding a polypeptide encoded by a sequence selected from the group consisting of SEQ ID NO: 78-86, 144, 145, 153, 167, 177, 193, 199, 205, 208, 215, 217, 220, 241, 242 246, 248, 249, 252, 256, 267, 270,

25 274, 277, 279, 282, 283, 285-287, 289 and 290.

Within another related aspect, the diagnostic kit comprises at least one oligonucleotide probe, the probe being specific for a polynucleotide described herein. In one embodiment, the probe comprises at least about 15 contiguous nucleotides of a polynucleotide as described above, or a polynucleotide selected from the group

30 consisting of SEQ ID NO: 78-86, 144, 145, 153, 167, 177, 193, 199, 205, 208, 215, 217,

220, 241, 242 246, 248, 249, 252, 256, 267, 270, 274, 277, 279, 282, 283, 285-287, 289 and 290.

In another related aspect, the present invention provides methods for monitoring the progression of breast cancer in a patient. In one embodiment, the method comprises: (a) detecting an amount, in a biological sample, of a polypeptide as described above at a first point in time; (b) repeating step (a) at a subsequent point in time; and (c) comparing the amounts of polypeptide detected in steps (a) and (b), and therefrom monitoring the progression of breast cancer in the patient. In another embodiment, the method comprises (a) detecting an amount, within a biological sample, of an RNA molecule encoding a polypeptide as described above at a first point in time; (b) repeating step (a) at a subsequent point in time; and (c) comparing the amounts of RNA molecules detected in steps (a) and (b), and therefrom monitoring the progression of breast cancer in the patient. In yet other embodiments, the present invention provides methods for monitoring the progression of breast cancer in a patient as described above wherein the polypeptide is encoded by a nucleotide sequence selected from the group consisting of SEQ ID NO: 78-86, 144, 145, 153, 167, 177, 193, 199, 205, 208, 215, 217, 220, 241, 242, 246, 248, 249, 252, 256, 267, 270, 274, 277, 279, 282, 283, 285-287, 289, 290 and sequences that hybridize thereto under stringent conditions.

In still other aspects, pharmaceutical compositions, which comprise a polypeptide as described above in combination with a physiologically acceptable carrier, and vaccines, which comprise a polypeptide as described above in combination with an immunostimulant or adjuvant, are provided. In yet other aspects, the present invention provides pharmaceutical compositions and vaccines comprising a polypeptide encoded by a nucleotide sequence selected from the group consisting of SEQ ID NO: 78-86, 144, 145, 153, 167, 177, 193, 199, 205, 208, 215, 217, 220, 241, 242 and 246, 248, 249, 252, 256, 267, 270, 274, 277, 279, 282, 283, 285-287, 289, 290 and sequences that hybridize thereto under stringent conditions.

In related aspects, the present invention provides methods for inhibiting the development of breast cancer in a patient, comprising administering to a patient a pharmaceutical composition or vaccine as described above.

These and other aspects of the present invention will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

5   BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the differential display PCR products, separated by gel electrophoresis, obtained from cDNA prepared from normal breast tissue (lanes 1 and 2) and from cDNA prepared from breast tumor tissue from the same patient (lanes 3 and 4). The arrow indicates the band corresponding to B18Ag1.

10   Figure 2 is a northern blot comparing the level of B18Ag1 mRNA in breast tumor tissue (lane 1) with the level in normal breast tissue.

Figure 3 shows the level of B18Ag1 mRNA in breast tumor tissue compared to that in various normal and non-breast tumor tissues as determined by RNase protection assays.

15   Figure 4 is a genomic clone map showing the location of additional retroviral sequences obtained from ends of XbaI restriction digests (provided in SEQ ID NO:3 - SEQ ID NO:10) relative to B18Ag1.

Figures 5A and 5B show the sequencing strategy, genomic organization and predicted open reading frame for the retroviral element containing B18Ag1.

20   Figure 6 shows the nucleotide sequence of the representative breast tumor-specific cDNA B18Ag1.

Figure 7 shows the nucleotide sequence of the representative breast tumor-specific cDNA B17Ag1.

Figure 8 shows the nucleotide sequence of the representative breast tumor-specific cDNA B17Ag2.

25   Figure 9 shows the nucleotide sequence of the representative breast tumor-specific cDNA B13Ag2a.

Figure 10 shows the nucleotide sequence of the representative breast tumor-specific cDNA B13Ag1b.

Figure 11 shows the nucleotide sequence of the representative breast tumor-specific cDNA B13Ag1a.

Figure 12 shows the nucleotide sequence of the representative breast tumor-specific cDNA B11Ag1.

Figure 13 shows the nucleotide sequence of the representative breast tumor-specific cDNA B3CA3c.

Figure 14 shows the nucleotide sequence of the representative breast tumor-specific cDNA B9CG1.

Figure 15 shows the nucleotide sequence of the representative breast tumor-specific cDNA B9CG3.

Figure 16 shows the nucleotide sequence of the representative breast tumor-specific cDNA B2CA2.

Figure 17 shows the nucleotide sequence of the representative breast tumor-specific cDNA B3CA1.

Figure 18 shows the nucleotide sequence of the representative breast tumor-specific cDNA B3CA2.

Figure 19 shows the nucleotide sequence of the representative breast tumor-specific cDNA B3CA3.

Figure 20 shows the nucleotide sequence of the representative breast tumor-specific cDNA B4CA1.

Figure 21A depicts RT-PCR analysis of breast tumor genes in breast tumor tissues (lanes 1-8) and normal breast tissues (lanes 9-13) and H_2O (lane 14).

Figure 21B depicts RT-PCR analysis of breast tumor genes in prostate tumors (lane 1, 2), colon tumors (lane 3), lung tumor (lane 4), normal prostate (lane 5), normal colon (lane 6), normal kidney (lane 7), normal liver (lane 8), normal lung (lane 9), normal ovary (lanes 10, 18), normal pancreases (lanes 11, 12), normal skeletal muscle (lane 13), normal skin (lane 14), normal stomach (lane 15), normal testes (lane 16), normal small intestine (lane 17), HBL-100 (lane 19), MCF-12A (lane 20), breast tumors (lanes 21-23), H_2O (lane 24), and colon tumor (lane 25).

Figure 22 shows the recognition of a B11Ag1 peptide (referred to as B11-8) by an anti-B11-8 CTL line.

Figure 23 shows the recognition of a cell line transduced with the antigen B11Ag1 by the B11-8 specific clone A1.

Figure 24 shows recognition of a lung adenocarcinoma line (LT-140-22) and a breast adenocarcinoma line (CAMA-1) by the B11-8 specific clone A1.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention is generally directed to compositions and methods for the diagnosis, monitoring and therapy of breast cancer. The compositions described herein include polypeptides, polynucleotides and antibodies. Polypeptides of the present invention generally comprise at least a portion of a protein that is expressed at a greater level in human breast tumor tissue than in normal breast tissue (*i.e.*, the level of RNA encoding the polypeptide is at least 2-fold higher in tumor tissue). Such polypeptides are referred to herein as breast tumor-specific polypeptides, and cDNA molecules encoding such polypeptides are referred to as breast tumor-specific cDNAs. Polynucleotides of the subject invention generally comprise a DNA or RNA sequence that encodes all or a portion of a polypeptide as described above, or that is complementary to such a sequence. Antibodies are generally immune system proteins, or fragments thereof, that are capable of binding to a portion of a polypeptide as described above. Antibodies can be produced by cell culture techniques, including the generation of monoclonal antibodies as described herein, or via transfection of antibody genes into suitable bacterial or mammalian cell hosts, in order to allow for the production of recombinant antibodies.

Polypeptides within the scope of this invention include, but are not limited to, polypeptides (and epitopes thereof) encoded by a human endogenous retroviral sequence, such as the sequence designated B18Ag1 (Figure 5 and SEQ ID NO:1). Also within the scope of the present invention are polypeptides encoded by other sequences within the retroviral genome containing B18Ag1 (SEQ ID NO: 141). Such sequences include, but are not limited to, the sequences recited in SEQ ID NO:3 - SEQ ID NO:10. B18Ag1 has homology to the *gag* p30 gene of the endogenous human retroviral element S71, as described in Werner et al., *Virology 174*:225-238 (1990) and also shows homology to about thirty other retroviral gag genes. As discussed in more detail below, the present invention also includes a number of additional breast tumor-

specific polypeptides, such as those encoded by the nucleotide sequences recited in SEQ ID NO: 11-26, 28-77, 142, 143, 146-152, 154-166, 168-176, 178-192, 194-198, 200-204, 206, 207, 209-214, 216, 218, 219, 221-240, 243-245, 247, 250, 251, 253, 255, 257-266, 268, 269, 271-273, 275, 276, 278, 280, 281, 284, 288, 291-298, 301-303, 307, 313, 314, 316 and 317.

As used herein, the term "polypeptide" encompasses amino acid chains of any length, including full length proteins containing the sequences recited herein. A polypeptide comprising an epitope of a protein containing a sequence as described herein may consist entirely of the epitope, or may contain additional sequences. The additional sequences may be derived from the native protein or may be heterologous, and such sequences may (but need not) possess immunogenic or antigenic properties.

An "epitope," as used herein is a portion of a polypeptide that is recognized (*i.e.*, specifically bound) by a B-cell and/or T-cell surface antigen receptor. Epitopes may generally be identified using well known techniques, such as those summarized in Paul, *Fundamental Immunology*, 3rd ed., 243-247 (Raven Press, 1993) and references cited therein. Such techniques include screening polypeptides derived from the native polypeptide for the ability to react with antigen-specific antisera and/or T-cell lines or clones. An epitope of a polypeptide is a portion that reacts with such antisera and/or T-cells at a level that is similar to the reactivity of the full length polypeptide (*e.g.*, in an ELISA and/or T-cell reactivity assay). Such screens may generally be performed using methods well known to those of ordinary skill in the art, such as those described in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. B-cell and T-cell epitopes may also be predicted via computer analysis. Polypeptides comprising an epitope of a polypeptide that is preferentially expressed in a tumor tissue (with or without additional amino acid sequence) are within the scope of the present invention.

The term "polynucleotide(s)," as used herein, means a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases and includes DNA and corresponding RNA molecules, including HnRNA and mRNA molecules, both sense and anti-sense strands, and comprehends cDNA, genomic DNA and recombinant DNA, as well as wholly or partially synthesized polynucleotides. An HnRNA molecule contains introns and corresponds to a DNA molecule in a generally one-to-one manner. An

mRNA molecule corresponds to an HnRNA and DNA molecule from which the introns have been excised. A polynucleotide may consist of an entire gene, or any portion thereof. Operable anti-sense polynucleotides may comprise a fragment of the corresponding polynucleotide, and the definition of "polynucleotide" therefore includes all such operable anti-sense fragments.

The compositions and methods of the present invention also encompass variants of the above polypeptides and polynucleotides.

A polypeptide "variant," as used herein, is a polypeptide that differs from the recited polypeptide only in conservative substitutions and/or modifications, such that the antigenic properties of the polypeptide are retained. In a preferred embodiment, variant polypeptides differ from an identified sequence by substitution, deletion or addition of five amino acids or fewer. Such variants may generally be identified by modifying one of the above polypeptide sequences, and evaluating the antigenic properties of the modified polypeptide using, for example, the representative procedures described herein. Polypeptide variants preferably exhibit at least about 70%, more preferably at least about 90% and most preferably at least about 95% identity (determined as described below) to the identified polypeptides.

As used herein, a "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. In general, the following groups of amino acids represent conservative changes: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his.

Variants may also, or alternatively, contain other modifications, including the deletion or addition of amino acids that have minimal influence on the antigenic properties, secondary structure and hydropathic nature of the polypeptide. For example, a polypeptide may be conjugated to a signal (or leader) sequence at the N-terminal end of the protein which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (e.g., poly-His), or to enhance

binding of the polypeptide to a solid support. For example, a polypeptide may be conjugated to an immunoglobulin Fc region.

A nucleotide "variant" is a sequence that differs from the recited nucleotide sequence in having one or more nucleotide deletions, substitutions or additions. Such modifications may be readily introduced using standard mutagenesis techniques, such as oligonucleotide-directed site-specific mutagenesis as taught, for example, by Adelman et al. (*DNA, 2*:183, 1983). Nucleotide variants may be naturally occurring allelic variants, or non-naturally occurring variants. Variant nucleotide sequences preferably exhibit at least about 70%, more preferably at least about 80% and most preferably at least about 90% identity (determined as described below) to the recited sequence.

The breast tumor antigens provided by the present invention include variants that are encoded by DNA sequences which are substantially homologous to one or more of the DNA sequences specifically recited herein. "Substantial homology," as used herein, refers to DNA sequences that are capable of hybridizing under moderately stringent conditions. Suitable moderately stringent conditions include prewashing in a solution of 5X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50°C-65°C, 5X SSC, overnight or, in the event of cross-species homology, at 45°C with 0.5X SSC; followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1% SDS. Such hybridizing DNA sequences are also within the scope of this invention, as are nucleotide sequences that, due to code degeneracy, encode an immunogenic polypeptide that is encoded by a hybridizing DNA sequence.

Two nucleotide or polypeptide sequences are said to be "identical" if the sequence of nucleotides or amino acid residues in the two sequences is the same when aligned for maximum correspondence as described below. Comparisons between two sequences are typically performed by comparing the sequences over a comparison window to identify and compare local regions of sequence similarity. A "comparison window" as used herein, refers to a segment of at least about 20 contiguous positions, usually 30 to about 75, 40 to about 50, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted using the Megalign program in the Lasergene suite of bioinformatics software (DNASTAR, Inc., Madison, WI), using default parameters. This program embodies several alignment schemes described in the following references: Dayhoff, M.O. (1978) A model of evolutionary change in proteins – Matrices for detecting distant relationships. In Dayhoff, M.O. (ed.) Atlas of Protein Sequence and Structure, National Biomedical Resarch Foundaiton, Washington DC Vol. 5, Suppl. 3, pp. 345-358; Hein J. (1990) Unified Approach to Alignment and Phylogenes pp. 626-645 *Methods in Enzymology* vol. 183, Academic Press, Inc., San Diego, CA; Higgins, D.G. and Sharp, P.M. (1989) Fast and sensitive multiple sequence alignments on a microcomputer *CABIOS 5*:151-153; Myers, E.W. and Muller W. (1988) Optimal alignments in linear space *CABIOS 4*:11-17; Robinson, E.D. (1971) *Comb. Theor 11*:105; Santou, N. Nes, M. (1987) The neighbor joining method. A new method for reconstructing phylogenetic trees *Mol. Biol. Evol. 4*:406-425; Sneath, P.H.A. and Sokal, R.R. (1973) *Numerical Taxonomy – the Principles and Practice of Numerical Taxonomy*, Freeman Press, San Francisco, CA; Wilbur, W.J. and Lipman, D.J. (1983) Rapid similarity searches of nucleic acid and protein data banks *Proc. Natl. Acad., Sci. USA 80*:726-730.

Preferably, the "percentage of sequence identity" is determined by comparing two optimally aligned sequences over a window of comparison of at least 20 positions, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e. gaps) of 20 percent or less, usually 5 to 15 percent, or 10 to 12 percent, as compared to the reference sequences (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid bases or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the reference sequence (i.e. the window size) and multiplying the results by 100 to yield the percentage of sequence identity. In general, polynucleotides encoding all or a portion of the polypeptides described herein may be prepared using any of several techniques. For example, cDNA molecules encoding such polypeptides may be cloned on the basis of the breast tumor-specific expression of the corresponding mRNAs, using differential display PCR. This technique compares the amplified

products from RNA template prepared from normal and breast tumor tissue. cDNA may be prepared by reverse transcription of RNA using a (dT)$_{12}$AG primer. Following amplification of the cDNA using a random primer, a band corresponding to an amplified product specific to the tumor RNA may be cut out from a silver stained gel and

5   subcloned into a suitable vector (*e.g.*, the T-vector, Novagen, Madison, WI). Polynucleotides encoding all or a portion of the breast tumor-specific polypeptides disclosed herein may be amplified from cDNA prepared as described above using the random primers shown in SEQ ID NO.:87-125.

Alternatively, a polynucleotide encoding a polypeptide as described

10   herein (or a portion thereof) may be amplified from human genomic DNA, or from breast tumor cDNA, via polymerase chain reaction. For this approach, B18Ag1 sequence-specific primers may be designed based on the sequence provided in SEQ ID NO:1, and may be purchased or synthesized. One suitable primer pair for amplification from breast tumor cDNA is (5'ATG GCT ATT TTC GGG GGC TGA CA) (SEQ ID NO:126) and

15   (5'CCG GTA TCT CCT CGT GGG TAT T) (SEQ ID NO:127). An amplified portion of B18Ag1 may then be used to isolate the full length gene from a human genomic DNA library or from a breast tumor cDNA library, using well known techniques, such as those described in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY (1989). Other sequences within the

20   retroviral genome of which B18Ag1 is a part may be similarly prepared by screening human genomic libraries using B18Ag1-specific sequences as probes. Nucleotides translated into protein from the retroviral genome shown in SEQ ID NO: 141 may then be determined by cloning the corresponding cDNAs, predicting the open reading frames and cloning the appropriate cDNAs into a vector containing a viral promoter, such as T7.

25   The resulting constructs can be employed in a translation reaction, using techniques known to those of skill in the art, to identify nucleotide sequences which result in expressed protein. Similarly, primers specific for the remaining breast tumor-specific polypeptides described herein may be designed based on the nucleotide sequences provided in SEQ ID NO:11-86, 142-298, 301-303, 307, 313, 314, 316 and 317.

30   Recombinant polypeptides encoded by the DNA sequences described above may be readily prepared from the DNA sequences. For example, supernatants

from suitable host/vector systems which secrete recombinant protein or polypeptide into culture media may be first concentrated using a commercially available filter. Following concentration, the concentrate may be applied to a suitable purification matrix such as an affinity matrix or an ion exchange resin. Finally, one or more reverse phase HPLC steps

5   can be employed to further purify a recombinant polypeptide.

In general, any of a variety of expression vectors known to those of ordinary skill in the art may be employed to express recombinant polypeptides of this invention. Expression may be achieved in any appropriate host cell that has been transformed or transfected with an expression vector containing a polynucleotide that

10   encodes a recombinant polypeptide. Suitable host cells include prokaryotes, yeast and higher eukaryotic cells. Preferably, the host cells employed are *E. coli*, yeast or a mammalian cell line such as COS or CHO.

Such techniques may also be used to prepare polypeptides comprising epitopes or variants of the native polypeptides. For example, variants of a native

15   polypeptide may generally be prepared using standard mutagenesis techniques, such as oligonucleotide-directed site-specific mutagenesis, and sections of the DNA sequence may be removed to permit preparation of truncated polypeptides. Portions and other variants having fewer than about 100 amino acids, and generally fewer than about 50 amino acids, may also be generated by synthetic means, using techniques well known to

20   those of ordinary skill in the art. For example, such polypeptides may be synthesized using any of the commercially available solid-phase techniques, such as the Merrifield solid-phase synthesis method, where amino acids are sequentially added to a growing amino acid chain. *See* Merrifield, *J. Am. Chem. Soc. 85*:2149-2146 (1963). Equipment for automated synthesis of polypeptides is commercially available from suppliers such as

25   Perkin Elmer/Applied BioSystems Division,, Foster City, CA, and may be operated according to the manufacturer's instructions.

In specific embodiments, polypeptides of the present invention encompass amino acid sequences encoded by a polynucleotide having a sequence recited in any one of SEQ ID NO:1, 3-26, 28-77, 142, 143, 146-152, 154-166, 168-176, 178-192, 194-198,

30   200-204, 206, 207, 209-214, 216, 218, 219, 221-240, 243-245, 247, 250, 251, 253, 255, 257-266, 268, 269, 271-273, 275, 276, 278, 280, 281, 284, 288, 291-298, 301-303, 307,

313, 314, 316 and 317, and variants of such polypeptides. Polypeptides within the scope of the present invention also include polypeptides (and epitopes thereof) encoded by DNA sequences that hybridize to a sequence recited in any one of SEQ ID NO:1, 3-26, 28-77, 142, 143, 146-152, 154-166, 168-176, 178-192, 194-198, 200-204, 206, 207, 209-

5 214, 216, 218, 219, 221-240, 243-245, 247, 250, 251, 253, 255, 257-266, 268, 269, 271-273, 275, 276, 278, 280, 281, 284, 288, 291-298, 301-303, 307, 313, 314, 316 and 317 under stringent conditions, wherein the DNA sequences are at least 80% identical in overall sequence to a recited sequence and wherein RNA corresponding to the nucleotide sequence is expressed at a greater level in human breast tumor tissue than in normal

10 breast tissue. As used herein, "stringent conditions" refers to prewashing in a solution of 6X SSC, 0.2% SDS; hybridizing at 65°C, 6X SSC, 0.2% SDS overnight; followed by two washes of 30 minutes each in 1X SSC, 0.1% SDS at 65°C and two washes of 30 minutes each in 0.2 X SSC, 0.1% SDS at 65°C. Polynucleotides according to the present invention include molecules that encode any of the above polypeptides.

15 In another aspect of the present invention, antibodies are provided. Such antibodies may be prepared by any of a variety of techniques known to those of ordinary skill in the art. *See, e.g.,* Harlow and Lane, *Antibodies: A Laboratory Manual,* Cold Spring Harbor Laboratory, 1988. In one such technique, an immunogen comprising the polypeptide is initially injected into any of a wide variety of mammals (*e.g.,* mice, rats,

20 rabbits, sheep or goats). In this step, the polypeptides of this invention may serve as the immunogen without modification. Alternatively, particularly for relatively short polypeptides, a superior immune response may be elicited if the polypeptide is joined to a carrier protein, such as bovine serum albumin or keyhole limpet hemocyanin. The immunogen is injected into the animal host, preferably according to a predetermined

25 schedule incorporating one or more booster immunizations, and the animals are bled periodically. Polyclonal antibodies specific for the polypeptide may then be purified from such antisera by, for example, affinity chromatography using the polypeptide coupled to a suitable solid support.

Monoclonal antibodies specific for the antigenic polypeptide of interest may be prepared, for example, using the technique of Kohler and Milstein, *Eur. J.*

30 *Immunol.* 6:511-519 (1976), and improvements thereto. Briefly, these methods involve

the preparation of immortal cell lines capable of producing antibodies having the desired specificity (*i.e.*, reactivity with the polypeptide of interest). Such cell lines may be produced, for example, from spleen cells obtained from an animal immunized as described above. The spleen cells are then immortalized by, for example, fusion with a

5   myeloma cell fusion partner, preferably one that is syngeneic with the immunized animal. A variety of fusion techniques may be employed. For example, the spleen cells and myeloma cells may be combined with a nonionic detergent for a few minutes and then plated at low density on a selective medium that supports the growth of hybrid cells, but not myeloma cells. A preferred selection technique uses HAT (hypoxanthine,

10   aminopterin, thymidine) selection. After a sufficient time, usually about 1 to 2 weeks, colonies of hybrids are observed. Single colonies are selected and their culture supernatants tested for binding activity against the polypeptide. Hybridomas having high reactivity and specificity are preferred.

Monoclonal antibodies may be isolated from the supernatants of growing

15   hybridoma colonies. In addition, various techniques may be employed to enhance the yield, such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and

20   extraction. The polypeptides of this invention may be used in the purification process in, for example, an affinity chromatography step.

Antibodies may be used, for example, in methods for detecting breast cancer in a patient. Such methods involve using an antibody to detect the presence or absence of a breast tumor-specific polypeptide as described herein in a suitable biological

25   sample. As used herein, suitable biological samples include tumor or normal tissue biopsy, mastectomy, blood, lymph node, serum or urine samples, or other tissue, homogenate, or extract thereof obtained from a patient.

There are a variety of assay formats known to those of ordinary skill in the art for using an antibody to detect polypeptide markers in a sample. *See, e.g.*, Harlow

30   and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. For example, the assay may be performed in a Western blot format, wherein a protein

preparation from the biological sample is submitted to gel electrophoresis, transferred to a suitable membrane and allowed to react with the antibody. The presence of the antibody on the membrane may then be detected using a suitable detection reagent, as described below.

In another embodiment, the assay involves the use of antibody immobilized on a solid support to bind to the polypeptide and remove it from the remainder of the sample. The bound polypeptide may then be detected using a second antibody or reagent that contains a reporter group. Alternatively, a competitive assay may be utilized, in which a polypeptide is labeled with a reporter group and allowed to bind to the immobilized antibody after incubation of the antibody with the sample. The extent to which components of the sample inhibit the binding of the labeled polypeptide to the antibody is indicative of the reactivity of the sample with the immobilized antibody, and as a result, indicative of the concentration of polypeptide in the sample.

The solid support may be any material known to those of ordinary skill in the art to which the antibody may be attached. For example, the solid support may be a test well in a microtiter plate or a nitrocellulose filter or other suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic material such as polystyrene or polyvinylchloride. The support may also be a magnetic particle or a fiber optic sensor, such as those disclosed, for example, in U.S. Patent No. 5,359,681.

The antibody may be immobilized on the solid support using a variety of techniques known to those in the art, which are amply described in the patent and scientific literature. In the context of the present invention, the term "immobilization" refers to both noncovalent association, such as adsorption, and covalent attachment (which may be a direct linkage between the antigen and functional groups on the support or may be a linkage by way of a cross-linking agent). Immobilization by adsorption to a well in a microtiter plate or to a membrane is preferred. In such cases, adsorption may be achieved by contacting the antibody, in a suitable buffer, with the solid support for a suitable amount of time. The contact time varies with temperature, but is typically between about 1 hour and 1 day. In general, contacting a well of a plastic microtiter plate (such as polystyrene or polyvinylchloride) with an amount of antibody ranging

from about 10 ng to about 1 µg, and preferably about 100-200 ng, is sufficient to immobilize an adequate amount of polypeptide.

Covalent attachment of antibody to a solid support may also generally be achieved by first reacting the support with a bifunctional reagent that will react with both

5   the support and a functional group, such as a hydroxyl or amino group, on the antibody. For example, the antibody may be covalently attached to supports having an appropriate polymer coating using benzoquinone or by condensation of an aldehyde group on the support with an amine and an active hydrogen on the binding partner (*see, e.g.*, Pierce Immunotechnology Catalog and Handbook (1991) at A12-A13).

10   In certain embodiments, the assay for detection of polypeptide in a sample is a two-antibody sandwich assay. This assay may be performed by first contacting an antibody that has been immobilized on a solid support, commonly the well of a microtiter plate, with the biological sample, such that the polypeptide within the sample are allowed to bind to the immobilized antibody. Unbound sample is then removed from

15   the immobilized polypeptide-antibody complexes and a second antibody (containing a reporter group) capable of binding to a different site on the polypeptide is added. The amount of second antibody that remains bound to the solid support is then determined using a method appropriate for the specific reporter group.

More specifically, once the antibody is immobilized on the support as

20   described above, the remaining protein binding sites on the support are typically blocked. Any suitable blocking agent known to those of ordinary skill in the art, such as bovine serum albumin or Tween 20™ (Sigma Chemical Co., St. Louis, MO). The immobilized antibody is then incubated with the sample, and polypeptide is allowed to bind to the antibody. The sample may be diluted with a suitable diluent, such as phosphate-buffered

25   saline (PBS) prior to incubation. In general, an appropriate contact time (*i.e.*, incubation time) is that period of time that is sufficient to detect the presence of polypeptide within a sample obtained from an individual with breast cancer. Preferably, the contact time is sufficient to achieve a level of binding that is at least 95% of that achieved at equilibrium between bound and unbound polypeptide. Those of ordinary skill in the art will

30   recognize that the time necessary to achieve equilibrium may be readily determined by

assaying the level of binding that occurs over a period of time. At room temperature, an incubation time of about 30 minutes is generally sufficient.

Unbound sample may then be removed by washing the solid support with an appropriate buffer, such as PBS containing 0.1% Tween 20™. The second antibody, which contains a reporter group, may then be added to the solid support. Preferred reporter groups include enzymes (such as horseradish peroxidase), substrates, cofactors, inhibitors, dyes, radionuclides, luminescent groups, fluorescent groups and biotin. The conjugation of antibody to reporter group may be achieved using standard methods known to those of ordinary skill in the art.

The second antibody is then incubated with the immobilized antibody-polypeptide complex for an amount of time sufficient to detect the bound polypeptide. An appropriate amount of time may generally be determined by assaying the level of binding that occurs over a period of time. Unbound second antibody is then removed and bound second antibody is detected using the reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For radioactive groups, scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups and fluorescent groups. Biotin may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups may generally be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products.

To determine the presence or absence of breast cancer, the signal detected from the reporter group that remains bound to the solid support is generally compared to a signal that corresponds to a predetermined cut-off value established from non-tumor tissue. In one preferred embodiment, the cut-off value is the average mean signal obtained when the immobilized antibody is incubated with samples from patients without breast cancer. In general, a sample generating a signal that is three standard deviations above the predetermined cut-off value may be considered positive for breast cancer. In an alternate preferred embodiment, the cut-off value is determined using a Receiver Operator Curve, according to the method of Sackett et al., *Clinical Epidemiology: A Basic Science for Clinical Medicine*, p. 106-7 (Little Brown and Co., 1985). Briefly, in

this embodiment, the cut-off value may be determined from a plot of pairs of true positive rates (*i.e.*, sensitivity) and false positive rates (100%-specificity) that correspond to each possible cut-off value for the diagnostic test result. The cut-off value on the plot that is the closest to the upper left-hand corner (*i.e.*, the value that encloses the largest area) is the most accurate cut-off value, and a sample generating a signal that is higher than the cut-off value determined by this method may be considered positive. Alternatively, the cut-off value may be shifted to the left along the plot, to minimize the false positive rate, or to the right, to minimize the false negative rate. In general, a sample generating a signal that is higher than the cut-off value determined by this method is considered positive for breast cancer.

In a related embodiment, the assay is performed in a flow-through or strip test format, wherein the antibody is immobilized on a membrane, such as nitrocellulose. In the flow-through test, the polypeptide within the sample bind to the immobilized antibody as the sample passes through the membrane. A second, labeled antibody then binds to the antibody-polypeptide complex as a solution containing the second antibody flows through the membrane. The detection of bound second antibody may then be performed as described above. In the strip test format, one end of the membrane to which antibody is bound is immersed in a solution containing the sample. The sample migrates along the membrane through a region containing second antibody and to the area of immobilized antibody. Concentration of second antibody at the area of immobilized antibody indicates the presence of breast cancer. Typically, the concentration of second antibody at that site generates a pattern, such as a line, that can be read visually. The absence of such a pattern indicates a negative result. In general, the amount of antibody immobilized on the membrane is selected to generate a visually discernible pattern when the biological sample contains a level of polypeptide that would be sufficient to generate a positive signal in the two-antibody sandwich assay, in the format discussed above. Preferably, the amount of antibody immobilized on the membrane ranges from about 25 ng to about 1 μg, and more preferably from about 50 ng to about 1 μg. Such tests can typically be performed with a very small amount of biological sample.

The presence or absence of breast cancer in a patient may also be determined by evaluating the level of mRNA encoding a breast tumor-specific polypeptide as described herein within the biological sample (*e.g.*, a biopsy, mastectomy and/or blood sample from a patient) relative to a predetermined cut-off value. Such an evaluation may be achieved using any of a variety of methods known to those of ordinary skill in the art such as, for example, *in situ* hybridization and amplification by polymerase chain reaction.

For example, polymerase chain reaction may be used to amplify sequences from cDNA prepared from RNA that is isolated from one of the above biological samples. Sequence-specific primers for use in such amplification may be designed based on the sequences provided in any one of SEQ ID NO: 1, 11-86, 142-298 301-303, 307, 313, 314, 316 and 317, and may be purchased or synthesized. In the case of B18Ag1, as noted herein, one suitable primer pair is B18Ag1-2 (5'ATG GCT ATT TTC GGG GGC TGA CA) (SEQ ID NO:126) and B18Ag1-3 (5'CCG GTA TCT CCT CGT GGG TAT T) (SEQ ID NO:127). The PCR reaction products may then be separated by gel electrophoresis and visualized according to methods well known to those of ordinary skill in the art. Amplification is typically performed on samples obtained from matched pairs of tissue (tumor and non-tumor tissue from the same individual) or from unmatched pairs of tissue (tumor and non-tumor tissue from different individuals). The amplification reaction is preferably performed on several dilutions of cDNA spanning two orders of magnitude. A two-fold or greater increase in expression in several dilutions of the tumor sample as compared to the same dilution of the non-tumor sample is considered positive.

As used herein, the term "primer/probe specific for a polynucleotide" means an oligonucleotide sequence that has at least about 80% identity, preferably at least about 90% and more preferably at least about 95%, identity to the polynucleotide in question, or an oligonucleotide sequence that is anti-sense to a sequence that has at least about 80% identity, preferably at least about 90% and more preferably at least about 95%, identity to the polynucleotide in question. Primers and/or probes which may be usefully employed in the inventive diagnostic methods preferably have at least about 10-40 nucleotides. In a preferred embodiment, the polymerase chain reaction primers

comprise at least about 10 contiguous nucleotides of a polynucleotide that encodes one of the polypeptides disclosed herein or that is anti-sense to a sequence that encodes one of the polypeptides disclosed herein. Preferably, oligonucleotide probes for use in the inventive diagnostic methods comprise at least about 15 contiguous oligonucleotides of a polynucleotide that encodes one of the polypeptides disclosed herein or that is anti-sense to a sequence that encodes one of the polypeptides disclosed herein. Techniques for both PCR based assays and *in situ* hybridization assays are well known in the art.

Conventional RT-PCR protocols using agarose and ethidium bromide staining, while important in defining gene specificity, do not lend themselves to diagnostic kit development because of the time and effort required in making them quantitative (i.e., construction of saturation and/or titration curves), and their sample throughput. This problem is overcome by the development of procedures such as real time RT-PCR which allows for assays to be performed in single tubes, and in turn can be modified for use in 96 well plate formats. Instrumentation to perform such methodologies are available from Perkin Elmer/Applied Biosystems Division. Alternatively, other high throughput assays using labeled probes (e.g., digoxygenin) in combination with labeled (e.g., enzyme fluorescent, radioactive) antibodies to such probes can also be used in the development of 96 well plate assays.

In yet another method for determining the presence or absence of breast cancer in a patient, one or more of the breast tumor-specific polypeptides described may be used in a skin test. As used herein, a "skin test" is any assay performed directly on a patient in which a delayed-type hypersensitivity (DTH) reaction (such as swelling, reddening or dermatitis) is measured following intradermal injection of one or more polypeptides as described above. Such injection may be achieved using any suitable device sufficient to contact the polypeptide or polypeptides with dermal cells of the patient, such as a tuberculin syringe or 1 mL syringe. Preferably, the reaction is measured at least 48 hours after injection, more preferably 48-72 hours.

The DTH reaction is a cell-mediated immune response, which is greater in patients that have been exposed previously to a test antigen (*i.e.*, an immunogenic portion of a polypeptide employed, or a variant thereof). The response may measured visually, using a ruler. In general, a response that is greater than about 0.5 cm in diameter,

preferably greater than about 5.0 cm in diameter, is a positive response, indicative of breast cancer.

The breast tumor-specific polypeptides described herein are preferably formulated, for use in a skin test, as pharmaceutical compositions containing at least one polypeptide and a physiologically acceptable carrier, such as water, saline, alcohol, or a buffer. Such compositions typically contain one or more of the above polypeptides in an amount ranging from about 1 µg to 100 µg, preferably from about 10 µg to 50 µg in a volume of 0.1 mL. Preferably, the carrier employed in such pharmaceutical compositions is a saline solution with appropriate preservatives, such as phenol and/or Tween 80™.

In other aspects of the present invention, the progression and/or response to treatment of a breast cancer may be monitored by performing any of the above assays over a period of time, and evaluating the change in the level of the response (*i.e.*, the amount of polypeptide or mRNA detected or, in the case of a skin test, the extent of the immune response detected). For example, the assays may be performed every month to every other month for a period of 1 to 2 years. In general, breast cancer is progressing in those patients in whom the level of the response increases over time. In contrast, breast cancer is not progressing when the signal detected either remains constant or decreases with time.

In further aspects of the present invention, the compounds described herein may be used for the immunotherapy of breast cancer. In these aspects, the compounds (which may be polypeptides, antibodies or polynucleotides) are preferably incorporated into pharmaceutical compositions or vaccines. Pharmaceutical compositions comprise one or more such compounds and a physiologically acceptable carrier. Vaccines may comprise one or more such compounds in combination with an immunostimulant, such as an adjuvant or a liposome (into which the compound is incorporated). An immunostimulant may be any substance that enhances or potentiates an immune response (antibody and/or cell-mediated) to an exogenous antigen. Examples of immunostimulants include adjuvants, biodegradable microspheres (*e.g.*, polylactic galactide) and liposomes (into which the compound is incorporated; *see e.g.*, Fullerton, U.S. Patent No. 4,235,877). Vaccine preparation is generally described in, for example,

M.F. Powell and M.J. Newman, eds., "Vaccine Design (the subunit and adjuvant approach)," Plenum Press (NY, 1995). Pharmaceutical compositions and vaccines within the scope of the present invention may also contain other compounds, which may be biologically active or inactive. For example, one or more immunogenic portions of other tumor antigens may be present, either incorporated into a fusion polypeptide or as a separate compound, within the composition or vaccine.

Alternatively, a vaccine may contain DNA encoding one or more of the polypeptides as described above, such that the polypeptide is generated *in situ*. In such vaccines, the DNA may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems, bacteria and viral expression systems. Appropriate nucleic acid expression systems contain the necessary DNA sequences for expression in the patient (such as a suitable promoter and terminating signal). Bacterial delivery systems involve the administration of a bacterium (such as *Bacillus-Calmette-Guerrin*) that expresses an immunogenic portion of the polypeptide on its cell surface. In a preferred embodiment, the DNA may be introduced using a viral expression system (*e.g.*, vaccinia or other pox virus, retrovirus, or adenovirus), which may involve the use of a non-pathogenic (defective), replication competent virus. Techniques for incorporating DNA into such expression systems are well known to those of ordinary skill in the art. The DNA may also be "naked," as described, for example, in Ulmer et al., *Science 259*:1745-1749 (1993), and reviewed by Cohen, *Science 259*:1691-1692 (1993). The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads, which are efficiently transported into the cells.

While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will vary depending on the mode of administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable

microspheres (*e.g.*, polylactate polyglycolate) may also be employed as carriers for the pharmaceutical compositions of this invention.

Any of a variety of immunostimulants may be employed in the vaccines of this invention. For example, an adjuvant may be included. Most adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bortadella pertussis* or *Mycobacterium tuberculosis* derived proteins. Suitable adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ); AS-2 (SmithKline Beecham, Philadelphia, PA); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF or interleukin-2, -7, or -12, may also be used as adjuvants.

Within the vaccines provided herein, the adjuvant composition is preferably designed to induce an immune response predominantly of the Th1 type. High levels of Th1-type cytokines (*e.g.*, IFN-γ, TNFα, IL-2 and IL-12) tend to favor the induction of cell mediated immune responses to an administered antigen. In contrast, high levels of Th2-type cytokines (*e.g.*, IL-4, IL-5, IL-6 and IL-10) tend to favor the induction of humoral immune responses. Following application of a vaccine as provided herein, a patient will support an immune response that includes Th1- and Th2-type responses. Within a preferred embodiment, in which a response is predominantly Th1-type, the level of Th1-type cytokines will increase to a greater extent than the level of Th2-type cytokines. The levels of these cytokines may be readily assessed using standard assays. For a review of the families of cytokines, see Mosmann and Coffman, *Ann. Rev. Immunol. 7*:145-173, 1989.

Preferred adjuvants for use in eliciting a predominantly Th1-type response include, for example, a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL), together with an aluminum salt. MPL adjuvants are available from Corixa Corporation (Seattle, WA; *see* US Patent Nos.

4,436,727; 4,877,611; 4,866,034 and 4,912,094). CpG-containing oligonucleotides (in which the CpG dinucleotide is unmethylated) also induce a predominantly Th1 response. Such oligonucleotides are well known and are described, for example, in WO 96/02555 and WO 99/33488. Immunostimulatory DNA sequences are also described, for example,

5   by Sato et al., *Science 273*:352, 1996. Another preferred adjuvant is a saponin, preferably QS21 (Aquila Biopharmaceuticals Inc., Framingham, MA), which may be used alone or in combination with other adjuvants. For example, an enhanced system involves the combination of a monophosphoryl lipid A and saponin derivative, such as the combination of QS21 and 3D-MPL as described in WO 94/00153, or a less

10   reactogenic composition where the QS21 is quenched with cholesterol, as described in WO 96/33739. Other preferred formulations comprise an oil-in-water emulsion and tocopherol. A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil-in-water emulsion is described in WO 95/17210.

Other preferred adjuvants include Montanide ISA 720 (Seppic, France),

15   SAF (Chiron, California, United States), ISCOMS (CSL), MF-59 (Chiron), the SBAS series of adjuvants (*e.g.*, SBAS-2 or SBAS-4, available from SmithKline Beecham, Rixensart, Belgium), Detox (Ribi ImmunoChem Research Inc., Hamilton, MT), RC-529 (Ribi ImmunoChem Research Inc., Hamilton, MT) and Aminoalkyl glucosaminide 4-phosphates (AGPs).

20   Any vaccine provided herein may be prepared using well known methods that result in a combination of antigen, immunostimulant and a suitable carrier or excipient. The compositions described herein may be administered as part of a sustained release formulation (*i.e.*, a formulation such as a capsule, sponge or gel (composed of polysaccharides, for example) that effects a slow release of compound following

25   administration). Such formulations may generally be prepared using well known technology (*see, e.g.*, Coombes et al., *Vaccine 14*:1429-1438, 1996) and administered by, for example, oral, rectal or subcutaneous implantation, or by implantation at the desired target site. Sustained-release formulations may contain a polypeptide, polynucleotide or antibody dispersed in a carrier matrix and/or contained within a reservoir surrounded by

30   a rate controlling membrane.

Carriers for use within such formulations are biocompatible, and may also

be biodegradable; preferably the formulation provides a relatively constant level of active component release. Such carriers include microparticles of poly(lactide-co-glycolide), as well as polyacrylate, latex, starch, cellulose and dextran. Other delayed-release carriers include supramolecular biovectors, which comprise a non-liquid hydrophilic core (*e.g.*, a cross-linked polysaccharide or oligosaccharide) and, optionally, an external layer comprising an amphiphilic compound, such as a phospholipid (*see e.g.*, U.S. Patent No. 5,151,254 and PCT applications WO 94/20078, WO/94/23701 and WO 96/06638). The amount of active compound contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

Any of a variety of delivery vehicles may be employed within pharmaceutical compositions and vaccines to facilitate production of an antigen-specific immune response that targets tumor cells. Delivery vehicles include antigen presenting cells (APCs), such as dendritic cells, macrophages, B cells, monocytes and other cells that may be engineered to be efficient APCs. Such cells may, but need not, be genetically modified to increase the capacity for presenting the antigen, to improve activation and/or maintenance of the T cell response, to have anti-tumor effects *per se* and/or to be immunologically compatible with the receiver (*i.e.*, matched HLA haplotype). APCs may generally be isolated from any of a variety of biological fluids and organs, including tumor and peritumoral tissues, and may be autologous, allogeneic, syngeneic or xenogeneic cells.

Certain preferred embodiments of the present invention use dendritic cells or progenitors thereof as antigen-presenting cells. Dendritic cells are highly potent APCs (Banchereau and Steinman, *Nature 392*:245-25,1, 1998) and have been shown to be effective as a physiological adjuvant for eliciting prophylactic or therapeutic antitumor immunity (*see* Timmerman and Levy, *Ann. Rev. Med. 50*:507-529, 1999). In general, dendritic cells may be identified based on their typical shape (stellate *in situ*, with marked cytoplasmic processes (dendrites) visible *in vitro*), their ability to take up, process and present antigens with high efficiency and their ability to activate naïve T cell responses. Dendritic cells may, of course, be engineered to express specific cell-surface receptors or ligands that are not commonly found on dendritic cells *in vivo* or *ex vivo*,

and such modified dendritic cells are contemplated by the present invention. As an alternative to dendritic cells, secreted vesicles antigen-loaded dendritic cells (called exosomes) may be used within a vaccine (*see* Zitvogel et al., *Nature Med. 4*:594-600, 1998).

5   Dendritic cells and progenitors may be obtained from peripheral blood, bone marrow, tumor-infiltrating cells, peritumoral tissues-infiltrating cells, lymph nodes, spleen, skin, umbilical cord blood or any other suitable tissue or fluid. For example, dendritic cells may be differentiated *ex vivo* by adding a combination of cytokines such as GM-CSF, IL-4, IL-13 and/or TNFα to cultures of monocytes harvested from

10   peripheral blood. Alternatively, CD34 positive cells harvested from peripheral blood, umbilical cord blood or bone marrow may be differentiated into dendritic cells by adding to the culture medium combinations of GM-CSF, IL-3, TNFα, CD40 ligand, LPS, flt3 ligand and/or other compound(s) that induce differentiation, maturation and proliferation of dendritic cells.

15   Dendritic cells are conveniently categorized as "immature" and "mature" cells, which allows a simple way to discriminate between two well characterized phenotypes. However, this nomenclature should not be construed to exclude all possible intermediate stages of differentiation. Immature dendritic cells are characterized as APC with a high capacity for antigen uptake and processing, which correlates with the high

20   expression of Fcγ receptor and mannose receptor. The mature phenotype is typically characterized by a lower expression of these markers, but a high expression of cell surface molecules responsible for T cell activation such as class I and class II MHC, adhesion molecules (*e.g.,* CD54 and CD11) and costimulatory molecules (*e.g.,* CD40, CD80, CD86 and 4-1BB).

25   APCs may generally be transfected with a polynucleotide encoding a polypeptide of the present invention (or portion or other variant thereof) such that the polypeptide, or an immunogenic portion thereof, is expressed on the cell surface. Such transfection may take place *ex vivo*, and a composition or vaccine comprising such transfected cells may then be used for therapeutic purposes, as described herein.

30   Alternatively, a gene delivery vehicle that targets a dendritic or other antigen presenting cell may be administered to a patient, resulting in transfection that occurs *in vivo. In vivo*

and *ex vivo* transfection of dendritic cells, for example, may generally be performed using any methods known in the art, such as those described in WO 97/24447, or the gene gun approach described by Mahvi et al., *Immunology and cell Biology 75*:456-460, 1997. Antigen loading of dendritic cells may be achieved by incubating dendritic cells or

5 progenitor cells with the polypeptide, DNA (naked or within a plasmid vector) or RNA; or with antigen-expressing recombinant bacterium or viruses (*e.g.,* vaccinia, fowlpox, adenovirus or lentivirus vectors). Prior to loading, the polypeptide may be covalently conjugated to an immunological partner that provides T cell help (*e.g.,* a carrier molecule). Alternatively, a dendritic cell may be pulsed with a non-conjugated

10 immunological partner, separately or in the presence of the polypeptide.

Vaccines and pharmaceutical compositions may be presented in unit-dose or multi-dose containers, such as sealed ampoules or vials. Such containers are preferably hermetically sealed to preserve sterility of the formulation until use. In general, formulations may be stored as suspensions, solutions or emulsions in oily or

15 aqueous vehicles. Alternatively, a vaccine or pharmaceutical composition may be stored in a freeze-dried condition requiring only the addition of a sterile liquid carrier immediately prior to use.

The above pharmaceutical compositions and vaccines may be used, for example, for the therapy of breast cancer in a patient. As used herein, a "patient" refers

20 to any warm-blooded animal, preferably a human. A patient may or may not be afflicted with breast cancer. Accordingly, the above pharmaceutical compositions and vaccines may be used to prevent the development of breast cancer or to treat a patient afflicted with breast cancer. In a preferred embodiment, the compounds are administered either prior to or following surgical removal of primary tumors and/or treatment by

25 administration of radiotherapy and conventional chemotherapeutic drugs. To prevent or slow the development of breast cancer, a pharmaceutical composition or vaccine comprising one or more polypeptides as described herein may be administered to a patient. Alternatively, naked DNA or plasmid or viral vector encoding the polypeptide may be administered. For treating a patient with breast cancer, the pharmaceutical

30 composition or vaccine may comprise one or more polypeptides, antibodies or

polynucleotides complementary to DNA encoding a polypeptide as described herein (*e.g.*, antisense RNA or antisense deoxyribonucleotide oligonucleotides).

Routes and frequency of administration, as well as dosage, will vary from individual to individual. In general, the pharmaceutical compositions and vaccines may be administered by injection (*e.g.*, intracutaneous, intramuscular, intravenous or subcutaneous), intranasally (*e.g.*, by aspiration) or orally. Between 1 and 10 doses may be administered for a 52-week period. Preferably, 6 doses are administered, at intervals of 1 month, and booster vaccinations may be given periodically thereafter. Alternate protocols may be appropriate for individual patients. A suitable dose is an amount of a compound that, when administered as described above, is capable of promoting an anti-tumor immune response. Such response can be monitored by measuring the anti-tumor antibodies in a patient or by vaccine-dependent generation of cytolytic effector cells capable of killing the patient's tumor cells *in vitro*. Such vaccines should also be capable of causing an immune response that leads to an improved clinical outcome (*e.g.*, more frequent remissions, complete or partial or longer disease-free survival) in vaccinated patients as compared to non-vaccinated patients. In general, for pharmaceutical compositions and vaccines comprising one or more polypeptides, the amount of each polypeptide present in a dose ranges from about 100 μg to 5 mg. Suitable dose sizes will vary with the size of the patient, but will typically range from about 0.1 mL to about 5 mL.

Polypeptides disclosed herein may also be employed in adoptive immunotherapy for the treatment of cancer. Adoptive immunotherapy may be broadly classified into either active or passive immunotherapy. In active immunotherapy, treatment relies on the *in vivo* stimulation of the endogenous host immune system to react against tumors with the administration of immune response-modifying agents (for example, tumor vaccines, bacterial adjuvants, and/or cytokines).

In passive immunotherapy, treatment involves the delivery of biologic reagents with established tumor-immune reactivity (such as effector cells or antibodies) that can directly or indirectly mediate antitumor effects and does not necessarily depend on an intact host immune system. Examples of effector cells include T lymphocytes (for example, CD8+ cytotoxic T-lymphocyte, CD4+ T-helper, tumor-infiltrating lymphocytes), killer cells (Natural Killer cells, lymphokine-activated killer cells), B

cells, or antigen presenting cells (such as dendritic cells and macrophages) expressing the disclosed antigens. The polypeptides disclosed herein may also be used to generate antibodies or anti-idiotypic antibodies (as in U.S. Patent No. 4,918,164), for passive immunotherapy.

5   The predominant method of procuring adequate numbers of T-cells for adoptive immunotherapy is to grow immune T-cells *in vitro*. Culture conditions for expanding single antigen-specific T-cells to several billion in number with retention of antigen recognition *in vivo* are well known in the art. These *in vitro* culture conditions typically utilize intermittent stimulation with antigen, often in the presence of cytokines,

10   such as IL-2, and non-dividing feeder cells. As noted above, the immunoreactive polypeptides described herein may be used to rapidly expand antigen-specific T cell cultures in order to generate sufficient number of cells for immunotherapy. In particular, antigen-presenting cells, such as dendritic, macrophage or B-cells, may be pulsed with immunoreactive polypeptides or transfected with a polynucleotide sequence(s), using

15   standard techniques well known in the art. For cultured T-cells to be effective in therapy, the cultured T-cells must be able to grow and distribute widely and to survive long term *in vivo*. Studies have demonstrated that cultured T-cells can be induced to grow *in vivo* and to survive long term in substantial numbers by repeated stimulation with antigen supplemented with IL-2 (see, for example, Cheever et al. *Ibid*).

20   The polypeptides disclosed herein may also be employed to generate and/or isolate tumor-reactive T-cells, which can then be administered to the patient. In one technique, antigen-specific T-cell lines may be generated by *in vivo* immunization with short peptides corresponding to immunogenic portions of the disclosed polypeptides. The resulting antigen specific CD8+ CTL clones may be isolated from the

25   patient, expanded using standard tissue culture techniques, and returned to the patient.

Alternatively, peptides corresponding to immunogenic portions of the polypeptides may be employed to generate tumor reactive T cell subsets by selective *in vitro* stimulation and expansion of autologous T cells to provide antigen-specific T cells which may be subsequently transferred to the patient as described, for example, by

30   Chang et al. *(Crit. Rev. Oncol. Hematol., 22*(3), 213, 1996).

In another embodiment, syngeneic or autologous dendritic cells may be pulsed with peptides corresponding to at least an immunogenic portion of a polypeptide

disclosed herein. The resulting antigen-specific dendritic cells may either be transferred into a patient, or employed to stimulate T cells to provide antigen-specific T cells which may, in turn, be administered to a patient. The use of peptide-pulsed dendritic cells to generate antigen-specific T cells and the subsequent use of such antigen-specific T cells

5   to eradicate tumors in a murine model has been demonstrated by Cheever et al. ("Therapy With Cultured T Cells: Principles Revisited," *Immunological Reviews, 157*:177, 1997).

Additionally vectors expressing the disclosed polynucleotides may be introduced into stem cells taken from the patient and clonally propagated *in vitro* for autologous

10   transplant back into the same patient. In one embodiment, cells of the immune system, such as T cells, may be isolated from the peripheral blood of a patient, using a commercially available cell separation system, such as CellPro Incorporated's (Bothell, WA) CEPRATE™ system (see U.S. Patent No. 5,240,856; U.S. Patent No. 5,215,926; WO 89/06280; WO 91/16116 and WO 92/07243). The separated cells are stimulated

15   with one or more of the immunoreactive polypeptides contained within a delivery vehicle, such as a microsphere, to provide antigen-specific T cells. The population of tumor antigen-specific T cells is then expanded using standard techniques and the cells are administered back to the patient.

20   The following Examples are offered by way of illustration and not by way of limitation.

EXAMPLES

25   EXAMPLE 1

PREPARATION OF BREAST TUMOR-SPECIFIC CDNAS USING

DIFFERENTIAL DISPLAY RT-PCR

This Example illustrates the preparation of cDNA molecules encoding

30   breast tumor-specific polypeptides using a differential display screen.

A.   Preparation of B18Ag1 cDNA and Characterization of mRNA Expression

Tissue samples were prepared from breast tumor and normal tissue of a patient with breast cancer that was confirmed by pathology after removal from the patient. Normal RNA and tumor RNA was extracted from the samples and mRNA was

5   isolated and converted into cDNA using a $(dT)_{12}AG$ (SEQ ID NO:130) anchored 3' primer. Differential display PCR was then executed using a randomly chosen primer (CTTCAACCTC) (SEQ ID NO:103). Amplification conditions were standard buffer containing 1.5 mM $MgCl_2$, 20 pmol of primer, 500 pmol dNTP, and 1 unit of *Taq* DNA polymerase (Perkin-Elmer, Branchburg, NJ). Forty cycles of amplification were

10   performed using 94°C denaturation for 30 seconds, 42°C annealing for 1 minute, and 72° C extension for 30 seconds. An RNA fingerprint containing 76 amplified products was obtained. Although the RNA fingerprint of breast tumor tissue was over 98% identical to that of the normal breast tissue, a band was repeatedly observed to be specific to the RNA fingerprint pattern of the tumor. This band was cut out of a silver stained gel,

15   subcloned into the T-vector (Novagen, Madison, WI) and sequenced.

The sequence of the cDNA, referred to as B18Ag1, is provided in SEQ ID NO:1. A database search of GENBANK and EMBL revealed that the B18Ag1 fragment initially cloned is 77% identical to the endogenous human retroviral element S71, which is a truncated retroviral element homologous to the Simian Sarcoma Virus (SSV). S71

20   contains an incomplete *gag* gene, a portion of the *pol* gene and an LTR-like structure at the 3' terminus (*see* Werner et al., *Virology 174*:225-238 (1990)). B18Ag1 is also 64% identical to SSV in the region corresponding to the P30 (gag) locus. B18Ag1 contains three separate and incomplete reading frames covering a region which shares considerable homology to a wide variety of gag proteins of retroviruses which infect

25   mammals. In addition, the homology to S71 is not just within the *gag* gene, but spans several kb of sequence including an LTR.

B18Ag1-specific PCR primers were synthesized using computer analysis guidelines. RT-PCR amplification (94°C, 30 seconds; 60°C → 42°C, 30 seconds; 72°C, 30 seconds for 40 cycles) confirmed that B18Ag1 represents an actual mRNA sequence

30   present at relatively high levels in the patient's breast tumor tissue. The primers used in amplification were B18Ag1-1 (CTG CCT GAG CCA CAA ATG) (SEQ ID NO:128) and

B18Ag1-4 (CCG GAG GAG GAA GCT AGA GGA ATA) (SEQ ID NO:129) at a

3.5 mM magnesium concentration and a pH of 8.5, and B18Ag1-2 (ATG GCT ATT TTC

GGG GCC TGA CA) (SEQ ID NO:126) and B18Ag1-3 (CCG GTA TCT CCT CGT

GGG TAT T) (SEQ ID NO:127) at 2 mM magnesium at pH 9.5. The same experiments

5   showed exceedingly low to nonexistent levels of expression in this patient's normal

breast tissue (see Figure 1). RT-PCR experiments were then used to show that B18Ag1

mRNA is present in nine other breast tumor samples (from Brazilian and American

patients) but absent in, or at exceedingly low levels in, the normal breast tissue

corresponding to each cancer patient. RT-PCR analysis has also shown that the B18Ag1

10   transcript is not present in various normal tissues (including lymph node, myocardium

and liver) and present at relatively low levels in PBMC and lung tissue. The presence of

B18Ag1 mRNA in breast tumor samples, and its absence from normal breast tissue, has

been confirmed by Northern blot analysis, as shown in Figure 2.

The differential expression of B18Ag1 in breast tumor tissue was also

15   confirmed by RNase protection assays. Figure 3 shows the level of B18Ag1 mRNA in

various tissue types as determined in four different RNase protection assays. Lanes 1-12

represent various normal breast tissue samples, lanes 13-25 represent various breast

tumor samples; lanes 26-27 represent normal prostate samples; lanes 28-29 represent

prostate tumor samples; lanes 30-32 represent colon tumor samples; lane 33 represents

20   normal aorta; lane 34 represents normal small intestine; lane 35 represents normal skin,

lane 36 represents normal lymph node; lane 37 represents normal ovary; lane 38

represents normal liver; lane 39 represents normal skeletal muscle; lane 40 represents a

first normal stomach sample, lane 41 represents a second normal stomach sample; lane

42 represents a normal lung; lane 43 represents normal kidney; and lane 44 represents

25   normal pancreas. Interexperimental comparison was facilitated by including a positive

control RNA of known β-actin message abundance in each assay and normalizing the

results of the different assays with respect to this positive control.

RT-PCR and Southern Blot analysis has shown the B18Ag1 locus to be

present in human genomic DNA as a single copy endogenous retroviral element. A

30   genomic clone of approximately 12-18 kb was isolated using the initial B18Ag1

sequence as a probe. Four additional subclones were also isolated by XbaI digestion.

Additional retroviral sequences obtained from the ends of the XbaI digests of these clones (located as shown in Figure 4) are shown as SEQ ID NO:3 - SEQ ID NO:10, where SEQ ID NO:3 shows the location of the sequence labeled 10 in Figure 4, SEQ ID NO:4 shows the location of the sequence labeled 11-29, SEQ ID NO:5 shows the location of the sequence labeled 3, SEQ ID NO:6 shows the location of the sequence labeled 6, SEQ ID NO:7 shows the location of the sequence labeled 12, SEQ ID NO:8 shows the location of the sequence labeled 13, SEQ ID NO:9 shows the location of the sequence labeled 14 and SEQ ID NO:10 shows the location of the sequence labeled 11-22.

Subsequent studies demonstrated that the 12-18 kb genomic clone contains a retroviral element of about 7.75 kb, as shown in Figures 5A and 5B. The sequence of this retroviral element is shown in SEQ ID NO: 141. The numbered line at the top of Figure 5A represents the sense strand sequence of the retroviral genomic clone. The box below this line shows the position of selected restriction sites. The arrows depict the different overlapping clones used to sequence the retroviral element. The direction of the arrow shows whether the single-pass subclone sequence corresponded to the sense or anti-sense strand. Figure 5B is a schematic diagram of the retroviral element containing B18Ag1 depicting the organization of viral genes within the element. The open boxes correspond to predicted reading frames, starting with a methionine, found throughout the element. Each of the six likely reading frames is shown, as indicated to the left of the boxes, with frames 1-3 corresponding to those found on the sense strand.

Using the cDNA of SEQ ID NO:1 as a probe, a longer cDNA was obtained (SEQ ID NO:227) which contains minor nucleotide differences (less than 1%) compared to the genomic sequence shown in SEQ ID NO:141.

B.   Preparation of cDNA Molecules Encoding Other Breast Tumor-Specific Polypeptides

Normal RNA and tumor RNA was prepared and mRNA was isolated and converted into cDNA using a $(dT)_{12}AG$ anchored 3' primer, as described above. Differential display PCR was then executed using the randomly chosen primers of SEQ ID NO: 87-125. Amplification conditions were as noted above, and bands observed to be specific to the RNA fingerprint pattern of the tumor were cut out of a silver stained

gel, subcloned into either the T-vector (Novagen, Madison, WI) or the pCRII vector (Invitrogen, San Diego, CA) and sequenced. The sequences are provided in SEQ ID NO:11 - SEQ ID NO:86. Of the 79 sequences isolated, 67 were found to be novel (SEQ ID NO:11-26 and 28-77) (*see also* Figures 6-20).

An extended DNA sequence (SEQ ID NO: 290) for the antigen B15Ag1 (originally identified partial sequence provided in SEQ ID NO: 27) was obtained in further studies. Comparison of the sequence of SEQ ID NO: 290 with those in the gene bank as described above, revealed homology to the known human β-A activin gene. Further studies led to the isolation of the full-length cDNA sequence for the antigen B21GT2 (also referred to as B311D; originally identified partial cDNA sequence provided in SEQ ID NO: 56). The full-length sequence is provided in SEQ ID NO: 307, with the corresponding amino acid sequence being provided in SEQ ID NO: 308. Further studies led to the isolation of a splice variant of B311D. The B311D clone of SEQ ID NO: 316 was sequenced and a XhoI/NotI fragment from this clone was gel purified and 32P-cDTP labeled by random priming for use as a probe for further screening to obtain additional B311D gene sequence. Two fractions of a human breast tumor cDNA bacterial library were screened using standard techniques. One of the clones isolated in this manner yielded additional sequence which includes a poly A+ tail. The determined cDNA sequence of this clone (referred to as B311D_BT1_1A) is provided in SEQ ID NO: 317. The sequences of SEQ ID NO: 316 and 317 were found to share identity over a 464 bp region, with the sequences diverging near the poly A+ sequence of SEQ ID NO: 317.

Subsequent studies identified an additional 146 sequences (SEQ ID NOS:142-289), of which 115 appeared to be novel (SEQ ID NOS:142, 143, 146-152, 154-166, 168-176, 178-192, 194-198, 200-204, 206, 207, 209-214, 216, 218, 219, 221-240, 243-245, 247, 250, 251, 253, 255, 257-266, 268, 269, 271-273, 275, 276, 278, 280, 281, 284, 288 and 291). To the best of the inventors' knowledge none of the previously identified sequences have heretofore been shown to be expressed at a greater level in human breast tumor tissue than in normal breast tissue.

In further studies, several different splice forms of the antigen B11Ag1 (also referred to as B305D) were isolated, with each of the various splice forms

containing slightly different versions of the B11Ag1 coding frame. Splice junction sequences define individual exons which, in various patterns and arrangements, make up the various splice forms. Primers were designed to examine the expression pattern of each of the exons using RT-PCR as described below. Each exon was found to show the same expression pattern as the original B11Ag1 clone, with expression being breast tumor-, normal prostate- and normal testis-specific. The determined cDNA sequences for the isolated protein coding exons are provided in SEQ ID NO: 292-298, respectively. The predicted amino acid sequences corresponding to the sequences of SEQ ID NO: 292 and 298 are provided in SEQ ID NO: 299 and 300. Additional studies using rapid amplification of cDNA ends (RACE), a 5' specific primer to one of the splice forms of B11Ag1 provided above and a breast adenocarcinoma, led to the isolation of three additional, related, splice forms referred to as isoforms B11C-15, B11C-8 and B11C-9,16. The determined cDNA sequences for these isoforms are provided in SEQ ID NO: 301-303, with the corresponding predicted amino acid sequences being provided in SEQ ID NO: 304-306.

In subsequent studies on B305D isoform A (cDNA sequence provided in SEQ ID NO: 292), the cDNA sequence (provided in SEQ ID NO: 313) was found to contain an additional guanine residue at position 884, leading to a frameshift in the open reading frame. The determined DNA sequence of this ORF is provided in SEQ ID NO: 314. This frameshift generates a protein sequence (provided in SEQ ID NO: 315) of 293 amino acids that contains the C-terminal domain common to the other isoforms of B305D but that differs in the N-terminal region.

EXAMPLE 2

PREPARATION OF B18AG1 DNA FROM HUMAN GENOMIC DNA

This Example illustrates the preparation of B18Ag1 DNA by amplification from human genomic DNA.

B18Ag1 DNA may be prepared from 250 ng human genomic DNA using 20 pmol of B18Ag1 specific primers, 500 pmol dNTPS and 1 unit of *Taq* DNA polymerase (Perkin Elmer, Branchburg, NJ) using the following amplification

parameters: 94°C for 30 seconds denaturing, 30 seconds 60°C to 42°C touchdown annealing in 2°C increments every two cycles and 72°C extension for 30 seconds. The last increment (a 42°C annealing temperature) should cycle 25 times. Primers were selected using computer analysis. Primers synthesized were B18Ag1-1, B18Ag1-2, B18Ag1-3, and B18Ag1-4. Primer pairs that may be used are 1+3, 1+4, 2+3, and 2+4.

Following gel electrophoresis, the band corresponding to B18Ag1 DNA may be excised and cloned into a suitable vector.

EXAMPLE 3

PREPARATION OF B18AG1 DNA FROM BREAST TUMOR CDNA

This Example illustrates the preparation of B18Ag1 DNA by amplification from human breast tumor cDNA.

First strand cDNA is synthesized from RNA prepared from human breast tumor tissue in a reaction mixture containing 500 ng poly A+ RNA, 200 pmol of the primer $(T)_{12}AG$ (i.e., TTT TTT TTT TTT AG) (SEQ ID NO: 130), 1X first strand reverse transcriptase buffer, 6.7 mM DTT, 500 mmol dNTPs, and 1 unit AMV or MMLV reverse transcriptase (from any supplier, such as Gibco-BRL (Grand Island, NY)) in a final volume of 30 µl. After first strand synthesis, the cDNA is diluted approximately 25 fold and 1 µl is used for amplification as described in Example 2. While some primer pairs can result in a heterogeneous population of transcripts, the primers B18Ag1-2 (5'ATG GCT ATT TTC GGG GGC TGA CA) (SEQ ID NO: 126) and B18Ag1-3 (5'CCG GTA TCT CCT CGT GGG TAT T) (SEQ ID NO: 127) yield a single 151 bp amplification product.

EXAMPLE 4

IDENTIFICATION OF B-CELL AND T-CELL EPITOPES OF B18AG1

This Example illustrates the identification of B18Ag1 epitopes.

The B18Ag1 sequence can be screened using a variety of computer algorithms. To determine B-cell epitopes, the sequence can be screened for

hydrophobicity and hydrophilicity values using the method of Hopp, *Prog. Clin. Biol. Res. 172B*:367-77 (1985) or, alternatively, Cease et al., *J. Exp. Med. 164*:1779-84 (1986) or Spouge et al., *J. Immunol. 138*:204-12 (1987). Additional Class II MHC (antibody or B-cell) epitopes can be predicted using programs such as AMPHI (*e.g.*, Margalit et al., *J.*

5   *Immunol. 138*:2213 (1987)) or the methods of Rothbard and Taylor (*e.g.*, *EMBO J. 7*:93 (1988)).

Once peptides (15-20 amino acids long) are identified using these techniques, individual peptides can be synthesized using automated peptide synthesis equipment (available from manufacturers such as Perkin Elmer/Applied Biosystems

10   Division, Foster City, CA) and techniques such as Merrifield synthesis. Following synthesis, the peptides can used to screen sera harvested from either normal or breast cancer patients to determine whether patients with breast cancer possess antibodies reactive with the peptides. Presence of such antibodies in breast cancer patient would confirm the immunogenicity of the specific B-cell epitope in question. The peptides can

15   also be tested for their ability to generate a serologic or humoral immune in animals (mice, rats, rabbits, chimps etc.) following immunization *in vivo*. Generation of a peptide-specific antiserum following such immunization further confirms the immunogenicity of the specific B-cell epitope in question.

To identify T-cell epitopes, the B18Ag1 sequence can be screened using

20   different computer algorithms which are useful in identifying 8-10 amino acid motifs within the B18Ag1 sequence which are capable of binding to HLA Class I MHC molecules. (*see, e.g.*, Rammensee et al., *Immunogenetics 41*:178-228 (1995)). Following synthesis such peptides can be tested for their ability to bind to class I MHC using standard binding assays (*e.g.*, Sette et al., *J. Immunol. 153*:5586-92 (1994)) and more

25   importantly can be tested for their ability to generate antigen reactive cytotoxic T-cells following *in vitro* stimulation of patient or normal peripheral mononuclear cells using, for example, the methods of Bakker et al., *Cancer Res. 55*:5330-34 (1995); Visseren et al., *J. Immunol. 154*:3991-98 (1995); Kawakami et al., *J. Immunol. 154*:3961-68 (1995); and Kast et al., *J. Immunol. 152*:3904-12 (1994). Successful *in vitro* generation of T-

30   cells capable of killing autologous (bearing the same Class I MHC molecules) tumor cells following *in vitro* peptide stimulation further confirms the immunogenicity of the

B18Ag1 antigen. Furthermore, such peptides may be used to generate murine peptide and B18Ag1 reactive cytotoxic T-cells following *in vivo* immunization in mice rendered transgenic for expression of a particular human MHC Class I haplotype (Vitiello et al., *J. Exp. Med. 173*:1007-15 (1991).

5   A representative list of predicted B18Ag1 B-cell and T-cell epitopes, broken down according to predicted HLA Class I MHC binding antigen, is shown below:

Predicted Th Motifs (B-cell epitopes) (SEQ ID NOS.: 131-133)

SSGGRTFDDFHRYLLVGI

10   QGAAQKPINLSKXIEVVQGHDE

SPGVFLEHLQEAYRIYTPFDLSA

Predicted HLA A2.1 Motifs (T-cell epitopes) (SEQ ID NOS.: 134-140)

YLLVGIQGA

15   GAAQKPINL

NLSKXIEVV

EVVQGHDES

HLQEAYRIY

NLAFVAQAA

20   FVAQAAPDS

EXAMPLE 5

IDENTIFICATION OF T-CELL EPITOPES OF B11AG1

This Example illustrates the identification of B11Ag1 (also referred to as

25   B305D) epitopes. Four peptides, referred to as B11-8, B11-1, B11-5 and B11-12 (SEQ ID NO: 309-312, respectfully) were derived from the B11Ag1 gene.

Human CD8 T cells were primed *in vitro* to the peptide B11-8 using dendritic cells according to the protocol of Van Tsai et al. (*Critical Reviews in Immunology 18*:65-75, 1998). The resulting CD8 T cell cultures were tested for their

30   ability to recognize the B11-8 peptide or a negative control peptide, presented by the B-LCL line, JY. Briefly, T cells were incubated with autologous monocytes in the presence

of 10 ug/ml peptide, 10 ng/ml IL-7 and 10 ug/ml IL-2, and assayed for their ability to specifically lyse target cells in a standard 51-Cr release assay. As shown in Fig. 22, the bulk culture line demonstrated strong recognition of the B11-8 peptide with weaker recognition of the peptide B11-1.

5   A clone from this CTL line was isolated following rapid expansion using the monoclonal antibody OKT3 and human IL-2. As shown in Fig. 23, this clone (referred to as A1), in addition to being able to recognize specific peptide, recognized JY LCL transduced with the B11Ag1 gene. This data demonstrates that B11-8 is a naturally processed epitope of the B11Ag1 gene. In addition these T cells were further found to

10   recognize and lyse, in an HLA-A2 restricted manner, an established tumor cell line naturally expressing B11Ag1 (Fig. 24). The T cells strongly recognize a lung adenocarcinoma (LT-140-22) naturally expressing B11Ag1 transduced with HLA-A2, as well as an A2+ breast carcinoma (CAMA-1) transduced with B11Ag1, but not untransduced lines or another negative tumor line (SW620).

15   These data clearly demonstrate that these human T cells recognize not only B11-specific peptides but also transduced cells, as well as naturally expressing tumor lines.

CTL lines raised against the antigens B11-5 and B11-12, using the procedures described above, were found to recognize corresponding peptide-coated

20   targets.

Example 6

CHARACTERIZATION OF BREAST TUMOR GENES DISCOVERED BY DIFFERENTIAL DISPLAY PCR

5   The specificity and sensitivity of the breast tumor genes discovered by differential display PCR were determined using RT-PCR. This procedure enabled the rapid evaluation of breast tumor gene mRNA expression semiquantitatively without using large amounts of RNA. Using gene specific primers, mRNA expression levels in a variety of tissues were examined, including 8 breast tumors, 5 normal breasts, 2 prostate

10   tumors, 2 colon tumors, 1 lung tumor, and 14 other normal adult human tissues, including normal prostate, colon, kidney, liver, lung, ovary, pancreas, skeletal muscle, skin, stomach and testes.

To ensure the semiquantitative nature of the RT-PCR, β-actin was used as internal control for each of the tissues examined. Serial dilutions of the first strand

15   cDNAs were prepared and RT-PCR assays performed using β-actin specific primers. A dilution was then selected that enabled the linear range amplification of β-actin template, and which was sensitive enough to reflect the difference in the initial copy number. Using this condition, the β-actin levels were determined for each reverse transcription reaction from each tissue. DNA contamination was minimized by DNase treatment and

20   by assuring a negative result when using first strand cDNA that was prepared without adding reverse transcriptase.

Using gene specific primers, the mRNA expression levels were determined in a variety of tissues. To date, 38 genes have been successfully examined by RT-PCR, five of which exhibit good specificity and sensitivity for breast tumors

25   (B15AG-1, B31GA1b, B38GA2a, B11A1a and B18AG1a). Figures 21A and 21B depict the results for three of these genes: B15AG-1 (SEQ ID NO:27), B31GA1b (SEQ ID NO:148) and B38GA2a (SEQ ID NO. 157). Table I summarizes the expression level of all the genes tested in normal breast tissue and breast tumors, and also in other tissues.

TABLE I

Percentage of Breast Cancer Antigens that are Expressed in Various Tissues

Breast Tissues	Over-expressed in Breast Tumors	84%
	Equally Expressed in Normals and Tumor	16%
Other Tissues	Over-expressed in Breast Tumors but not in any Normal Tissues	9%
	Over-expressed in Breast Tumors but Expressed in Some Normal Tissues	30%
	Over-expressed in Breast Tumors but Equally Expressed in All Other Tissues	61%

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for the purpose of illustration, various modifications may be made without deviating from the spirit and scope of the invention.